# 3-DIMENSIONAL *IN VITRO* MODELS OF MAMMALIAN TISSUES

## FIELD OF THE INVENTION

The present invention relates generally to *in vitro* models of mammalian tissues. More particularly, the invention relates to 3-dimensional *in vitro* models simulating the behavior of a cancerous human tissue at various stages of a solid tumour progression, to 3-dimensional *in vitro* model simulating the senescence and apoptosis of normal and cancerous human cells, and to the use of such models for drug screening and testing. The invention further relates to novel mathematical models for cell growth and drug toxicity data analysis and cell-cell coupling simulation.

## BACKGROUND OF THE INVENTION

Screening for bioactive chemical agents affecting in a desirable manner functions of human organs and tissues, particularly for agents useful for treating pathological conditions and disorders, is of great importance for the human well being and is becoming one of the fastest developing research areas. As human testing is subject to very stringent limitations, various models have been devised to simulate biological responses of human tissues to such chemical agents, for example to screen for new promising drugs.

Models presently in use to simulate biological responses to chemical agents include experimental animals, explanted tissue slices, monolayer cell cultures, and mathematical models based on the chemical structure of leader compounds. None of these models offers a full compatibility with *in vivo* human tissues and each has its advantages and shortcomings. Experimental animals are expensive to maintain and there are ethical considerations associated with the use of animals for such purposes. Moreover, because

the cells and tissues of animals are different from those of humans, test results are not always applicable to humans.

Monolayer cultures of human cells have the advantage of some biochemical similarity to human tissues and usually provide highly reproducible, inexpensive, and well standardized test systems. However, 2-dimensional (2D) cell cultures are not morphologically and physiologically similar to *in vivo* tissues and do not simulate well the state of cells and tissues in the organism, especially because they do not reproduce the cytoarchitecture found in the living organism. Furthermore, 2D cell cultures cannot reproduce the orderly structure found in tissues formed of two or more cell phenotypes. As a consequence, the biosynthetic activities and physiological functions expressed by cells grown in monolayer cultures are markedly different from those in the organism, and may frequently lead to misleading test findings.

The shortcomings of 2D cell cultures stimulated the development of various 3-dimensional (3D) models of human tissues, intended to simulate more closely the morphological and physiological characteristics of their *in vivo* counterparts. Examples of such 3D models are a 3D model of brain blood barrier (US 5,578,485) and a 3D tumour cell and tissue culture system (US 5,580,781).

3D models of human tissues known in the prior art frequently show a high degree of sophistication and morphological resemblance to their *in vivo* counterparts. However, such 3D structures are usually grown following complicated protocols and even though nominally useful for screening for agents with clinical utility, they are poorly adapted to high throughput screening procedures typical of the modern drug development, in particular for screening large combinatorial libraries of new compounds. There exists therefore a need for 3D models of human tissues combining the advantages of 3D tissue models and compatibility with the requirements of modern drug testing procedures. The present invention provides such models which are free of many prior art limitations.

## SUMMARY OF THE INVENTION

According to one aspect, the present invention provides novel 3-dimentional *in vitro* models of mammalian tissues. The models comprise 3-dimensional cell aggregates (organoids) grown from mammalian, preferably human cells of at least two different phenotypes in a suitable liquid growth medium.

In a preferred embodiment, the organoids are grown from various combinations of normal and tumour cells. These models are designed to simulate three stages of a solid tumour progression: promotion/angiogenesis, invasion and metastasis. In another preferred embodiment, the organoids are grown from two sub-populations of identical cells, one of which was pre-treated with a chemical agent modifying cell properties. There are two models in this group, designed to simulate cell senescence and cell apoptosis, respectively. The first model uses cells pre-treated with an agent which blocks cell proliferation without killing the cells, whereas the second uses cell modified with a phototoxic compound, which induces cell death upon illumination of cells.

The models have a large variety of applications, particularly in drug screening. These tests are based, for example, on measuring simultaneously and comparing proliferation rates of at least two different cell phenotypes in organoids cultured in the presence and in the absence of a candidate drug. In a preferred embodiment, proliferation rates are calculated from results of flow cytometry analysis of single cells in suspension obtained from the dispersion of the organoids, which cells were fluorescently labeled prior to forming the organoids.

Other advantages, objects and features of the present invention will be readily apparent to those skilled in the art from the following detailed description of preferred embodiments in conjunction with the accompanying drawings and claims.

## BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1A is a graph illustrating cell proliferation analysis in organoids of the present invention; the graph shows results of flow cytometry analysis of a fluorescently labeled single cell suspension obtained from dispersed organoids.

Fig. 1B is a graph showing the distribution of the subpopulation of cells that underwent 0, 1, 2, and 3 divisions, respectively, in four days old organoids.

Fig. 2A is a graph reporting the proliferation kinetics based on the proliferation index of Neuro2A neuroblastoma cells grown in ES/Neuro 2A cells aggregates containing increasing proportion of embryonic stem cells (ES).

Fig. 2B is a graph reporting the proliferation kinetics based on the proliferation index of the ES cells grown in the same ES/Neuro2A cell aggregates as those in Fig. 2B. This is an example of simultaneous proliferation assessment of two cell phenotypes co-cultured in the same 3D organoids.

Fig. 3 is a drawing representing schematically the design of a photoablation experiment.

Fig. 4 is a gallery of graphs showing the effects of increasing the percentage of photoablated cells in the spheroids from 0 to 75% (top to bottom) on the kinetics of the regeneration/proliferation rate over a 4 days period (left to right); the analyses were performed as illustrated in Fig. 1.

Fig. 5 is a gallery of graphs showing gap junction intercellular communication in spheroids containing various proportions of ES cells and mitomycin-treated ES cells; intercellular communication is measured by the number of cells to which calcein was transferred (receiver cells) from the donor cells.

Fig. 6 is a graph showing temporal changes of the proliferation index of ES cells in cell aggregates containing increasing number of mytomycin-treated ES cells; this is an example of simultaneous determination of the level of intercellular communication (Fig. 5) and cell-subpopulations flow cytometry analyses (Fig. 1) in a two-phenotype organoid.

Fig. 7 is a graph showing effect of a chemical (AGA) on the maintenance of a nonproliferative fraction (quiescent pool) in 3D embryoid bodies (EB) of embryonic stem cells (ES-EBs + AGA); comparison with the ES-EB control and with a hyperplastic cell line (F9-EBs).

Fig. 8 is a graph showing effect of AGA on the development of cell coupling in ES-EBs of two cell lines (ES and F9).

Fig. 9 is a graph showing kinetics of the cell recruitment rate from the quiescent pool of the ES-embryoid bodies of Fig. 7.

Fig. 10 is a graph showing kinetics of the cell recruitment rate from the quiescent pool of gap junction proficient and gap junction deficient embryoid bodies (EBs) of Fig. 8.

Fig. 11 is a graph showing simulated number of mitoses per 1000 cells in a 3D cell system over a period of 290 time units, with the heavy line showing its Fourier cosine transform.

Fig. 12 is a graph showing simulated number of cells in G0-t state per 100 cells in a 3D cell system over a period of 290 time units, with the heavy line showing its Fourier cosine transform.

## DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention provides 3D *in vitro* models for simulating various characteristics of mammalian tissues, in particular cancerous tissues. The models of the present invention are 3D aggregates (organoids) formed from living cells of mammalian origin. As used herein, the term "organoid" is intended to mean any 3-dimensional aggregation of living cells of at least two different phenotypes, which aggregation may be grown either in an organized, orderly fashion, or by a random association of cells, in the presence or absence of a solid support. Organoids may be sometimes referred to as "aggregates", particularly when grown from cells of a single phenotype. When characterized by an essentially spheroidal shape, organoids (aggregates) may be also referred to as "spheroids".

Organized 3D organoid structures for *in vitro* models according to the present invention may be formed, for example, by culturing cells on a solid support either of natural or artificial origin, under conditions favoring the growth of 3D cellular structures, for example, by growing different cell phenotypes in superposed layers on the support. Random organoid structures may be formed, for example, by co-culturing cells of different phenotypes stepwise, in the absence or presence of a solid support. It is also possible to grow suitable organoids by various combinations of unsupported or supported, organized or random cell culturing, according to methods well known to those skilled in the art. According to a preferred embodiment, organoids according to the present invention are grown in suspension in rotating culture flasks (spinner flasks), in the absence or presence of a solid support.

Cells of different phenotypes may be either cells of different mammalian tissues or cells of the same tissue having different phenotypic characteristics. In the latter case, the different phenotypic characteristics may be a result of either natural or artificial cell transformations, including but not limited to natural pathological cell transformations, in particular cancerous

transformations, genetic engineering, or treatment with chemical or physical agents. Examples of cells suitable for the growing of organoids according to the present invention include but are not limited to, stem cells, in particular embryonic stem cells, endothelial, stromal, neural, liver, kidney, bladder, prostate, skin and heart cells. Primary cells may be isolated from normal or pathological mammalian tissues, in particular from tumour tissues, or taken from a large variety of commercially available cells and immortalized or transformed cell lines, as well as various genetically engineered cells.

The *in vitro* models of the present invention are intended for studying various characteristics of normal and cancerous mammalian tissues, in particular for the assessment of potential anti-cancer drugs at three specific stages of progression of a solid tumour: promotion/angiogenesis, invasion, and metastasis. A model is also proposed for studying cell senescence and apoptosis and for the assessment of drugs promoting cell regeneration and tissue repair. The latter model may also be adapted to the study of drugs inducing cell differentiation or to preparing cells for xenotransplantation. As will be clear from the following, the applications of the models of the present invention go far beyond the assessment of anti-cancer drugs. Additional applications of the models will be discussed in connection with the preferred embodiments of the invention.

The studies and assessment of tissue functions and characteristics using the tissue models according to the present invention rely in most cases on identifying and quantifying sub-populations of cells (cell phenotypes) building the model organoids under study. This usually takes place after preparing such organoids from at least two sub-populations of cells of different phenotypes, culturing the organoids for a predetermined period of time under predetermined conditions, for example in the presence of a candidate drug, and dispersing the cultured organoids into a suspension of individual cells. The identification and counting of individual cells in the suspension is normally carried out by an automated method using appropriately marked or labeled cells. Examples of suitable markers and labels are fluorescent,

radioactive and immunospecific markers and labels, applied to the cells by techniques well known to those skilled in the art. The cells may be marked either prior to forming organoids or after dispersing cultured organoids into a suspension of individual cells.

According to a preferred embodiment of the invention, cells are marked or labeled fluorescently prior to forming organoids or aggregates and are identified and counted by flow cytometry (fluorescent analysis by cell sorting, or FACS) after the cultured organoids or aggregates are dispersed into a suspension of individual cells. The kind of the fluorescent labeling of cell sub-populations and specific markers or labels used depend on the property under study. For example, using fluorescent membrane linkers, such as PKH26 (red fluorescence) or PKH67 (green fluorescence), available from Sigma, is preferred for measuring cell proliferation, whereas loading cells with a fluorescent dye, such as calcein-AM is preferred when studying cell communication. Simultaneous evaluation of multiple sub-populations of cells of different phenotypes may be achieved by labeling cells of each sub-population with markers or labels fluorescing at different wavelengths.

Fluorescent membrane linkers, preferably used for the measuring of cell proliferation, allow the analysis of the distribution of cell populations which underwent 1, 2, 3, ..... n divisions. In this case, the proliferation of cells in the models according to the present invention is preferably measured by the proliferation index (PI). The proliferation index measures the ratio of the total number of cells of a given phenotype in the analyzed sample of cells to the calculated number of cells in the parent population at the time of formation of the organoid and can be expressed as:

$$PI = \frac{\sum_{k=0}^{n} A_k}{\sum_{k=0}^{n} \frac{A_k}{2^k}}$$

where $A_k$ represents the number of cells that underwent k divisions. The percentage of cells that underwent k = 1, 2, ..... n division can be estimated

from flow cytometry measurements, based on the fact that at each generation of daughter cells inherit only half of the fluorescence of the mother cell generation marked with a fluorescent membrane linker.

According to one preferred embodiment, the invention provides an *in vitro* model of angiogenesis in cancer. In this model, endothelial cells of mammalian origin labeled with a fluorescent marker are mixed in a known proportion with tumour cells and allowed to form spheroids. The cancer cells produce angiogenic growth factors which affect the growth rate of endothelial cells. The growth rate of endothelial cells can be measured and related directly to tumour angiogenesis.

In an alternative embodiment, the fluorescently labeled endothelial cells are allowed to adhere in a culture to microcarrier beads, such as cytodex or cytopore from Amersham Pharmacia Biotech Ltd. Once the beads are covered with endothelial cells, tumour cells are added to suspension of microbeads covered with endothelial cells and are allowed to adhere to the latter. The tumour cells/endothelial cells spheroids are then allowed to grow in a suitable culture medium. In a control experiment, similar organoids composed of endothelial cells and normal cells matching those of the tumour, e.g., normal urothelium cells in the case of bladder cancer, are prepared, grown and analyzed.

The model of angiogenesis in cancer is particularly useful for identifying and studying potential angiogenic drugs. By using organoids formed with endothelial and other normal cells, this model can be easily adapted to identifying and/or studying potential angiogenic agents, capable of inducing neovascularization in the process of tissue repair.

According to another preferred embodiment, the invention provides an *in vitro* model of interaction between stromal and tumour cells. In this model, fluorescently labeled normal stromal cells matching the tumour cells under study are grown as spheroids on microcarrier beads in a stepwise manner.

After the microbeads/stromal cells spheroids are formed, tumour cells fluorescently labeled with a marker different from that used to identify the stromal cells are added to the culture and allowed to adhere on the top of the stromal cell layer. The spheroids are then allowed to grow in suspension.

This model is particularly useful for studying the kinetics of anticancer drugs blocking the invasiveness of the tumour, for measuring the growth rate of both the stromal and tumour cells, for studying molecular interactions between stromal and tumour cells, for measuring the expression of markers of proliferation and differentiation, for measuring the level of gap junction mediated cell-cell communication, and for evaluating adhesion of tumour cells to the stromal cells.

According to another preferred embodiment, the invention provides an *in vitro* model of metastasis. In this model, epithelial or other cells representative of the tissue where metastases of a given tumour are expected to develop are grown as a monolayer (2D culture) on a lower surface of a porous solid membrane, such as a Milipore inset. Small spheroids formed in roller flasks from fluorescently labeled tumour cells are seeded on the upper surface of the membrane. Depending on the porosity of the membrane, the tumour cells infiltrate the membrane and establish direct contacts with the epithelial (or other) cells. For example, human normal lung fibroblasts can be used in the monolayer, with spheroids of appropriate fluorescently labeled tumour cells applied to the opposite side of the membrane. Both populations of cells may be fluorescently labeled if information on the growth or apoptosis of both populations is required, for example to see if the tumour cells recruit the quiescent cells to which they adhere and induce them to divide. This information may be needed to check the effect of a potential anticancer drug on the growth of a metastatic tumour and/or its intercellular communication with the cells to which it adheres.

The model of metastases is particularly useful for testing potential antimetastatic drugs, based on criteria such as the rate of growth of both

types of cells, communication between cells, and cell adhesion, invasion and/or differentiation.

According to another preferred embodiment, the invention provides an *in vitro* model of apoptosis, senescence, necrosis and tissue regeneration. In this model, normal cells of any origin can be used. For monitoring the effect of senescence, a given percentage of a single cell population is pre-treated with a chemical agent which blocks cell proliferation without killing the cells, such as mitomycin. The rest of the population is fluorescently labeled with a membrane linker and the two cell subpopulations are mixed and allowed to form spheroids. The regeneration of spheroids is followed by monitoring the proliferation of cells neighboring the growth-arrested or apoptotic cells.

This model can be easily adapted to monitoring the effects of apoptosis. In this case, the first subpopulation of cells is loaded with a phototoxic compound, such as chloromethyl eosine diacetate and the second one is fluorescently labeled. The two subpopulations are mixed and allowed to form spheroids which are then illuminated to induce cell death of cells loaded with the phototoxic compound. The regeneration of spheroids is followed by monitoring the proliferation of the surviving cells. The percentage of cells dying of apoptosis can be estimated by propidium iodide exposure and cell cycle analysis by FACS, a technique well known to those skilled in the art.

For this model, original protocols were developed to measure the effect of increasing the number of senescent, necrotic, or apoptotic cells on the proliferative ability of neighboring cells. The model is particularly useful for testing drugs potentiating regeneration in damaged tissues, especially in neurodegenerative diseases, for studying cell senescence and death, and to test for apoptosis inducers. When used for the latter purpose, the model can provide a positive control for testing drugs that induce apoptosis in solid tumours. In this case, an aliquot of the tumour cells is fluorescently labeled with a membrane linker and treated with the drug. After a predetermined period of time, the drug-treated cells are mixed in a given proportion with

untreated tumour cells. By comparison with the intreated subpopulation, efficacy of the drug, possible bystander effect, number of resistant cells and regrowth of resistant cells can be measured. When cells isolated from biopsies are used to form the spheroids, the model can also applied in association with the mathematical simulation models SIMCAN to predict the regrowth of the tumour after its irradiation (or chemotherapy) and hence the chance for such a treatment to succeed.

Various analytical procedures have been developed to assess results of tests when using the tissue models of the present invention. These include the following:

- measuring the diffusion rate of drugs, by tracking drugs labeled with a fluorescent tag, using either imaging microscopy or sequential mild trypsinization and flow cytometry (FACS);
- identification of cells' phenotypes and live cell sorting using membrane markers, in particular fluorescent membrane linkers;
- simultaneous assessment of proliferation kinetics of two cell sub-populations, by FACS analysis of the sub-populations previously labeled with fluorescent markers fluorescing at two different wavelengths; this technique allows very fine analysis of population growth at the single cell level; FACS data are analyzed with ModFit software (Sigma);
- measuring cell-cell communication by labeling one cell sub-population, prior to seeding, with a red-fluorescing membrane linker (PKH26, from Sigma) and loading the cells of the other sub-population with a green-fluorescing dye (calcein), which can only transfer between the cells through the gap junction channels;
- quantifying proliferation, differentiation and apoptotic cell markers by immunolabeling and flow cytometry.

The above procedures are based on principles and techniques well known to those skilled in the art.

Several computer simulation and data analysis routines have been developed in connection with the mammalian tissue models of the present invention. These include the following:

- PROFILE – for building a profile of a subpopulation susceptible to a given disease using an array of various attributes of individuals;
- AD-JUST - for finding the optimal fit of a theoretical function to a set of experimental data points;
- SIMCEL-2D – a 2D growth simulation model based on a 2D cellular automaton that mimics monolayer cell cultures with various degrees of gap junction intercellular communication; for predicting of growth kinetics and drug diffusion in monolayer cell cultures;
- SIMCEL-3D – a 3D growth simulation model based on a 3D cellular automaton with various levels of gap junction intercellular communication and mitotic and death rates; for predicting of growth kinetics in cell mass tissues, under conditions allowing for homeostatic behavior and for homeostasis disruption.

The AD-JUST routine is applicable, for example, in analysis of the growth-related parameters of *in vitro* models of the present invention and in analysis of drug toxicity data in both 2- and 3-dimensional tissue models.

The analysis of *in vitro* models of the present invention includes a routine estimation of cell-cell coupling. Intercellular communications mediated by gap junction channels are altered in most if not all cancers (such as breast, bladder, prostate, and lung cancer) and in many other diseases (neuropathologies, heart, lung, and kidney related illnesses, psoriasis, etc.). The proteins that form the channel (connexins) constitute a novel therapeutical target. The two simulation models (SIMCEL-2D and SIMCEL-3D) are used for the prediction of drug efficacy or gene therapies (for example, the bystander effect) based on the gap junction function. The estimation of intercellular coupling using these theoretical models, whose validity was tested in the in vitro models of the present invention, allows the

prediction of dosage and temporal efficacity of drugs designed to modulate the gap junction function.

A brief description of the above routines and the algorithms involved follows.

## PROFILE

### Goal

Build a profile of a sub-population susceptible to a given disease using an array of various attributes of individuals. Example: In a population we know a group of $N_1$ individuals who developed the disease. We also know $K$ attributes of these individuals with regard to various domains (life history, genetical, pathological antecedents, social, professional, etc). We have the same information for a random group of $N_0$ ($N_1$=$N_0$) individuals who did not developed the disease. The algorithm consists in finding out a unique combination of attributes that best predict the occurrence and non-occurrence of the disease. Attributes consists mostly in categorial and ordinal information. This model can also be applied to cell communities for which different attributes (such as growth parameters, phenotypes, pathology, disease stage, etc.) are known.

### Algorithm

The overall algorithm uses a basic module based on information theory.

*Basic module*

Let $f_{k,i,1}$ and $f_{k,i,0}$ be the number of individuals who respectively did and did not develop the disease in category $i$ of the *kth* attribute. Let $N = N_1 + N_0$ be the size of the whole sample. We derive:

$$H_k(0,1) = \frac{1}{N}\sum_{i=1}^{n}\left(f_{k,i}\ln f_{k,i} - f_{k,i,0}\ln f_{k,i,0} - f_{k,i,1}\ln f_{k,i,1}\right)$$

and

$$R_k = \left(\ln 2 - H_k(0,1)\right)/H(k)$$

with

$$H(k) = \frac{1}{N}\left( N \ln N - \sum_{i=1}^{n} f_{k,i} \ln f_{k,i} \right)$$

where $R_k$ measures the efficiency of attribute $k$ as a predictor of the occurrence/non-occurrence of the disease. It can be tested using the *maximum likelihood ratio*.

*Overall procedure*

The above module is embedded in an stepwise hierarchical divisive procedure. At each divisive step the attribute $k^*$ with the highest $R_k$ is selected as the divisive attribute and the sample of individuals subdivided according the categories of $k^*$. The subsets produced are then submitted to the same procedure

The divisive process goes on until anyone of following stopping rules applies: i) number of individuals too small in a subset to warrant test validity; ii) $H(0,1)<0.3$ in a subset (one of the 2 outcomes has a *0.9* probability; iii) no attribute still available exhibits a significant $R_k$.

The PROFILE routine is a novel adaptation of the PEGASE routine (Phipps, M. (1981), Entropy and Community Pattern Analysis, *Journal of Theoretical Biology* **93**: 253-273)
dealing with spatial pattern analysis. PROFILE, algorithm and routine, have been designed for population targeting for medical purposes and include a number of mathematical features which were not present in the former algorithm.

**AD-JUST**

**Goal**

Find out the optimal fit of a theoretical function to a set of experimental data points. These data may express time dependent responses, dose dependent responses, pseudo-cyclic time series, etc. This routine includes a set of theoretical functions: linear, exponential, reverse exponential, logistic, reverse logistic, polynomial functions and, in the case or time series the Fourier cosine transform and the exponential decreasing weigh averaging (EDWA). For each application all functions are tested and results are given for the 3 best $r^2$ fits.

**Algorithms**

*Linear*

*Exponential and reverse exponential*

This classical growth function is usually presented as

$$y = e^{ax+b}$$

or its equivalent form

$$y = Be^{ax}$$

with

$$B = e^{b}$$

We propose an improved calculus procedure by introducing a constant $c$ such

$$y = c + e^{ax+b}$$

as

which can be linearized as

$$\ln(y - c) = ax + b$$

Since *c* is not known, the AD-JUST routine finds its value by an optimization iterative process.

In the case of a reverse exponential, we have

$$y = c - e^{ax+b}$$

with

$$\ln(y + c) = ax + b$$

and the value of *c* is found using the same iterative procedure.

Compared with results obtained from similar commercial software available on the market, the goodness of fit correlation $r^2$ is considerably improved.

*Logistic and reverse logistic*

This classical population growth function is not commonly represented in commercial software packages. We propose an algorithm introducing an overall constant c in the basic logistic equation and an original linearization procedure.

Let *y* be the basic logistic or reverse logistic equation:

$$y = y_0 \pm \frac{1}{c + e^{-ax+b}}$$

We derive the linear form using

$$Y = y \pm y_0$$

and

$$\ln\left(\frac{1}{Y} - c\right) = -ax + b$$

Since *c* is not known its value is found using an optimizing iterative procedure and an initial value

$$c_0 = 1/Y_{max} + \varepsilon$$

where

$Y_{max}$

is the largest empirical value observed and ε is an infinitesimal quantity.

*Polynomial functions*

*Fourier cosine transform*

*Exponential decreasing weigh averaging*

This model applies to time series and can be a substitute to the Fourier cosine transform. Let $x_t$ (: $t = 1, T$ ) be a series of empirical values observed where T represents time or any other dimension like a spatial gradient. We want to smooth the graphic representation of this series by replacing observed values with calculated values eliminating erratic variations.

Let $v_t$ (: $t = 1, T$ ) be this substituted value. It is derived from $x_t$ using:

$$V_t = \left( \frac{V_{t-1}}{a} + x_t \right) \quad \text{and} \quad v_t = \frac{V_t}{1 + 1/(a-1)}$$

where $a > 1$ is an integer or decimal point number that allows for more or less smoothing. The smaller *a* the smoother the data series. Optimal *a* values fall in the range $1.1 < a < 1.5$ .

*Overall AD-JUST procedure*

The overall AD-JUST procedure consists of:

- preparing data (computation of statistical values, average, standard error, etc.);
- testing all available functions (computing the $r^2$ goodness of fit correlations);
- selecting the 3 best fits;
- producing graphs, and values tables.

**SIMCEL-2D and SIMCEL-3D**

SimCel-2D and SimCel-3D are two distinct cellular automaton (CA) models designed to simulate the dynamical behavior of 2-D and 3-D cells systems. Given that both share a number of common functions, they will be described under the same section heading. As the names indicate, they only differ in terms of their dimensionality and cell spatial arrangement.

**Algorithm, structure and functions**

*Cell network*

SimCel-2D is based on a 2-D regular hexagonal cell network (honey-comb like with a 1 to 6 contact ratio).

SimCel-3D is based on a 3-D regular cell network where cell are represented as regular dodecahedron arranged in a dense sphere packing system, with a 1 to 12 contact ratio.

*Accessible cell states*

Individual cells have access to a set of states split in 2 subsets (resting and divisive):

1. Within the resting group, cells go successively through:

- G0t state (resting with fully functional communication channels and capacity to resume proliferation). G0-tx is the same state without channels;
- G0-d state (differentiating resting cells with decreasing channels functionality and capacity to resume proliferation. G0-dx is the same state without channels;
- Dead cells.

2. Within the divisive group:

- S state (cell synthesizing DNA);
- G2-M state (cells completing genomic replication and mitosis);
- DC daughter cells issued from mitosis;

*Neighborhood*

Each cell *u* has a neighborhood that consists in its 6 adjacent cells in SimCel-2D and 12 adjacent cells in SimCel-3D.

*CA transition rule*

The transition from the resting group to the divisive group (i.e. G0 S) or vice versa from the divisive to the resting group (i.e. DC G0) is controlled by a probabilistic transition rule subject to a set of probabilities $p_{out}$ and $p_{1ut}$ that cell *u* at time *t adopts* G0 or S. Probabilities are updated at each time unit

$$p_{0u,t+1} = p_{0ut} + \mathrm{d}p_{ut} \quad \text{and} \quad p_{1u,t+1} = p_{1ut} - \mathrm{d}p_{ut}$$

according to

with:

$$\mathrm{d}p_{ut} = r\rho_{ut}k_{ut}\vartheta_{ut}\left[\left(a_{0ut}p_{0ut}\right) - \left(a_{1ut}p_{1ut}\right)\right] + \varepsilon\vartheta_{ut}k_{ut}$$

where *r* is an overall parameter controlling the intensity of cell-cell communication; $\rho_{ut}$ and $\vartheta_{ut}$ are age-dependent extinction factors, $a_{0ut}$ and $a_{1ut}$

are factors expressing neighborhood effects on the variation of both probabilities, $k_{ut}$ is a limit to this variation and $\varepsilon$ is a random Gaussian factor.

It should be noted that G0-tx and G0-dx cells which do not develop communication channels, $r$ is permanently set to 0, a mathematical condition equivalent to the lack of channels.

Cell state change from one group to the other (i.e. resting to divisive or vice-versa) subject to probabilities $p_{0ut}$ and $p_{1ut}$ and a pseudo random number.

Within each state groups (resting or divisive) cell change their state according to an age dependent process and fixed time sequences.

*Other functions*

SimCel-2D and -3D make provision for two additional functions: *mitosis* and *mortality*. Six time unit after entering the divisive cycle, a cycling cell produces 2 daughter cells and the additional new cell finds its place in the system by a cascade of centrifugal cell movements.

A cell in the resting group is assigned an age and is submitted to an age dependent probabilistic death program subject to a probability $\mu_{ut}$ such as:

$$\mu_{ut} = \beta e^{\alpha(\omega_{ut}-1)} \quad \text{with} \quad \alpha = \frac{\ln(1/0.005)}{\Omega - 1}$$

where $\Omega$ is the maximum cell life span and $\omega_{ut}$ is the age of cell $u$ at time $t$.

Following a decay period, a dead cell is removed from the system and replaced by a neighboring cell and a cascade of centripetal cell movements.

*Initial configuration*

At the onset of a simulation run, an initial configuration is given. By random choices, each cell is assigned a state, an age in its state subject to *a priori* frequencies, and a 0/1 (i.e. *yes-or-no*) capacity to communicate subject to probabilities $q_0$ and $q_1$ that represent basic characteristics of the simulated cell line.

*Routine outputs*

During the simulation run several dynamic graphic or numerical outputs are displayed:

- dynamic mapping of the cell system (for SimCel-3D an equatorial 2-D section is shown);
- dynamic analysis of state frequencies (G0-t, G0-d, S, G2M, Dead);
- dynamic graphic of cell probability;
- proliferation index;
- recruitment rate of cycling cells from the resting pool;

For the final configuration:

- assessment of the cell-to-cell intracellular diffusion (percolation) based on the average number of cells in the 10 longest strings of communicating cells.

**Previous publications on this subject**

Phipps, M. Dynamical Behavior of Cellular Automata under the Constraint of Neighborhood Coherence. *Geographical Analysis* **21**: 197-215 (1989).

Phipps, M., J. Darozewski & J. Phipps. How the Neighborhood Coherence Principle (NCP) Can Give Rise to Tissue Homeostasis. *Journal of Theoretical Biology* **185**: 475-487 (1997).

The present implementation extends the basic algorithm by providing dynamic graphic displays, the introduction of probabilities $q_0$ and $q_1$ determining an *a priori* cell capacity to communicate, and the assessment of the percolation-diffusion capacity in the cell system.

# EXAMPLES

## EXAMPLE 1

**Simultaneous measurement of proliferation and proliferation indexes in two phenotypes grown as aggregates**

Before forming aggregates, the two sub-populations (gap junction proficient embryonic stem cells (ES cells) and gap junction deficient neuroblastoma cells (Neuro2A cells) were labeled with the red membrane linker PKH26 and yellow membrane linker PKH67 (Sigma), respectively, according to modified manufacturer instructions. The cells were then mixed in known proportions and allowed to form aggregates that were sampled every day for monitoring growth. For analysis, the aggregates were dissociated to single cell suspensions using appropriate technique suited to the cell type analyzed. It is known that at each division the parent cell loses half of its fluorescence intensity. The individual cell fluorescence of no less than 10,000 cells was measured by flow cytometry and the results analyzed using ModFit software (Sigma). The control showing the mean fluorescence of the cell populations homogeneously labeled served as the reference first peak (Fig. 1A). The data illustrate one such experiment. The first peak measures the label at time 0. The graphs obtained each day measure the distribution of the population into subpopulations of cells having divided 1, 2, 3, ..... n times. Each successive peak from right to left represents the percentage of cells forming each subpopulation (Fig. 1B). The proliferation index is calculated from these data.

Fig. 2A shows that the growth (measured by the proliferation index) of one cell phenotype is not affected by the percentage of co-cultured cells in the aggregates. In contrast, the proliferation of the second phenotype is dependent on the number of cells of the second phenotype present in the aggregates (Fig. 2B). Cell-cell communication was also simultaneously measured in this experiment and controls of possible growth factor secretion by the phenotypes were included (results not shown).

EXAMPLE 2

**Photoablation**

Photoablation was achieved using the chloromethyl eosin diacetate (CMEDA, Molecular Probe). A suspension of individual cells was loaded with a solution of 20 μM of CMEDA in acetoxymethylester buffer according to modified manufacturer's instructions, for 30 minutes at 37°C, using a 5 mM stock solution of CMEDA in dimethylsulphoxide (DMSO).

Cells were washed twice with the growth medium, mixed in known proportions with untreated cells labeled with the PKH26 membrane linker and allowed to form spheroids. The spheroids were then illuminated at a 30 cm distance with an incandescent lamp source for 30 min in phosphate buffered saline. The illumination caused the death of the CMEDA loaded cells by individual photoablation (Fig. 3).

The regeneration of the organoids following the photoablation was quantified by the calculated proliferative index and the number of cell divisions. The results are summarized in Fig. 4. The figure shows the effects of increasing the percentage of photoablated cells from 0 to 75% (top to bottom) in the spheroids on the kinetics of the regeneration/proliferation rate over a 4 days period (left to right). The data show that the larger the percentage of dead cells, the higher the proliferation/regeneration cell rate.

EXAMPLE 3

**Senescent Model**

Embryonic stem (ES) cells (D3 strain) were grown as monolayer in Dulbecco modified Earle medium (DMEM) containing 15% ES certified fetal bovine serum (FBS) and 1000 U/ml LIF. Actively proliferating cells were harvested by

trypsinization and resuspended as a single cell suspension. One half of a single cell suspension was exposed to mitomycin C for one hour (optimum time for ES cells) using a 1mg/ml stock solution of mytomycin. Mitomycin is a DNA intercalating agent that irreversibly blocks the cell cycle and DNA synthesis at appropriate concentration. The cells which pass the restriction point complete the cycle. Mitomycin also blocks the tyrosine kinases. The cells remain alive but metabolically inactive and display features of senescent cells.

The second half of the cell suspension (untreated) was labeled with the membrane linker PKH26 which fluoresces in red. The cell suspensions were then mixed at increasing ratio of mitomycin treated versus PKH26 labeled cells (25, 50, and 75%) and allowed to form embryoid bodies (3D spheroids) in 3.5 cm bacterial plates. Controls embryoid bodies contained 0% mitomycin treated cells. In an independent experiment, it was checked that mitomycin-treated cells aggregate to form spheroids, but do not proliferate. Gap junction intercellular communication (GJIC) and the proliferation of the ES cells in mixed spheroids were measured as follows.

GJIC measured by dye transfer

The mytomycin–treated cells are extensively communicating, as illustrated by Fig. 5. The assay was performed as follows. A mytomycin-treated subpopulation of ES cells was loaded with calcein, a cell-permeant dye which fluoresces in green after de-esterification by intracellular esterases. The subpopulation of calcein-loaded ES cells (donor cells) was mixed with the untreated ES cells (receiver cells) labeled on the membrane with the red fluorescent membrane linker PKH26 (at 75/25, 50/50 and 25/75 ratio) and allowed to form spheroids. After being de-esterified, calcein becomes impermeant and can only diffuse to the unloaded cells through the gap junction channels. In this population of cells, receiver cells fluoresce red, donor cells (calcein-loaded) fluoresce green and the communicating cells fluoresce green and red (yellow). Cells were analyzed at time 0 and 24 hrs following disruption of spheroids to a single cell suspension. The results

obtained from flow cytometry analysis (FACS) are shown in graphs A through E of Fig. 5.

The PKH26-labeled cells show in the quadrant 1 and the calcein-loaded cells appear in the quadrant 4. The cells that fluoresce both red and green are found in the quadrant 2. From A to E, the graphs show the red-fluorescent population, the green-fluorescent population (both at time 0), then the mixed populations after 24 hrs. The ratio of untreated ES cells to mitomycin-treated ES cells is indicated at the top of each graph.

The levels of dye transfer, measured in the quadrant 2, are the following:

| | | |
|---|---|---|
| A2 | 1.7 | ES/ESmito: 100/0 |
| B2 | 0.1 | ES/ESmito: 0/100 |
| C2 | 75.2 | ES/ESmito: 75/25 |
| D2 | 58.6 | ES/ESmito: 50/50 |
| E2 | 29.7 | ES/ESmito: 25/75 |

It can be seen from the above that:

1. A2 and B2 measure background since the cells fluoresce either red or green. No dye transfer has occurred at time 0.
2. C2 through E2 levels represent the number of cells in which the dye transfer occurred from mitomycin–treated ES cells to untreated ES cells in percent.

All the ES cells have received the calcein dye. The results clearly show that mitomycin-treated cells are GJIC competent and communicate with their untreated counterparts.

Proliferation analyses

The data are acquired on the PKH26-labeled, untreated ES cells following trypsinization of spheroids to single cell suspension and FACS analysis. The

number of cells at 1, 2, 3..... generations is expressed as a percentage of the sample population analyzed by FACS (usually 10,000 cells).

At 24 hrs, all the spheroids containing mitomycin-treated cells already show a second peak representative of cells that underwent one division, while the matching control does not. Therefore after 24 hrs cells in spheroids containing only normal (untreated) ES cells did not divide. In contrast, 3.3 to 7.15% of the cells from spheroids containing 25 to 75%, respectively, of senescent (mitomycin-treated) cells entered the cell cycle.

At 48 hrs, the second generation in controls reaches 55.92%. The spheroids containing treated cells exhibit a third generation. The proliferative index increases with the increasing amount of treated cells in the spheroids. The proliferation data at 48 and 96 hrs are summarized in Tables 1 and 2.

Table 1. Effect of increasing percentage of senescent cells on the regeneration rate at 48 hrs

| Generations | Control | 75/25 | 50/50 | 25/75 |
|---|---|---|---|---|
| Gen. 1 | 43.78 | 40.86 | 21.18 | 20.34 |
| Gen. 2 | 55.92 | 50.53 | 52.65 | 46.91 |
| Gen. 3 | 0.00 | 8.12 | 26.15 | 32.62 |

Table 2. Effect of increasing percentages of senescent cells on the regeneration rate at 96 hrs

| Generations | Control | 75/25 | 50/50 | 25/75 |
|---|---|---|---|---|
| Gen. 1 | 19.40 | 20.47 | 13.40 | 5.63 |
| Gen. 2 | 44.19 | 39.71 | 28.87 | 16.58 |
| Gen. 3 | 29.23 | 32.41 | 35.47 | 30.00 |
| Gen. 4 | 6.27 | 6.04 | 21.96 | 29.26 |
| Gen. 5 | 0.66 | 1.09 | 0.13 | 18.49 |

In the above tables, the first number in the ratio corresponds to the percentage of untreated ES cells. The results are summarized in Fig. 6 using proliferation index data.

It is clear from these results that the proliferation rate of the normal cells increases in the presence of senescent (mitomycin-treated) cells when compared to control and that the increase is directly proportional to the proportion of the senescent cells in the spheroids. As a whole, the results show that:

1. cell replacement is promoted by dead or apoptotic (as in Fig. 4) or metabolically inactive cells (as in this experiment);
2. cell proliferation is correlated with the number of dead cells;
3. cell number in a delineated compartment (here organoids) is regulated; and
4. gap junctions contribute to all processes, probably by translocating signaling molecules.

EXAMPLE 4

**Model of angiogenesis in bladder cancer**

Cell lines used:

Human endothelial cells from Wisent Company (Wisent, St Bruno, Canada);

RT4 cell line (ATCC HTB2), derived from well differentiated human papillary bladder carcinoma;

J82 cell line (ATCC HTB1), derived from poorly differentiated invasive bladder carcinoma;

FHs-738B1 cells (ATCC HTB 160), derived from human normal urothelium.

Two single cell sub-populations, endothelial and either one of RT4 and J82 or the control FHs-738B1 cells, were mixed in a known proportion and allowed to form organoids in spinner flasks in an appropriate medium. The cancer cells produce angiogenic growth factors. The factors reflect on the growth rate of endothelial cells and can be directly related to tumour angiogenesis.

The growth rate of endothelial cells were tested using a fluorescent membrane linker, PKH26 (Sigma, USA), as described in Example 1. The number of endothelial cells can also be monitored using the immuno-detection of CD31 and CD34 (a membrane marker of PECAM-1 and microvessels, respectively). Gap Junction Intercellular Communication (GJIC) was quantitatively estimated as described in Example 3. In addition, the CD34 immunopositive endothelial cells can be sorted out alive using FACS technology for further microscopic studies.

The diffusion rate of potential drugs added to the medium was measured by adding a fluorescent tag to the drug molecule and tracking the molecule by video-microscopy imaging. Putative positive effect of the formation of microvasculature from the endothelial cells on the penetration of nutrients inside the spheroids was measured using a classical fluorescent doxorubicin diffusion test.

Alternatively, embryonic stem cells having formed embryoid bodies that contain microvessels were co-cultured with single cell populations of either one of the bladder cancer cell lines to form spheroids and were analyzed as described above.

In another version of the model, endothelial cells were grown on microcarriers containing slowly released blood substitute. This extends the use of the model from small, non-vascularized microtumours or metastases to larger oxygenated tumours containing "functional" microvessels.

In this model, any cancer cell phenotype can be substituted for bladder cell lines.

EXAMPLE 5

**Model of interaction between stromal and tumour cells.**

The HCV-29 cell line of normal urothelium fibroblastic cells was used to form the stromal layer for the preparation of mixed spheroids. The normal stromal cells HCV-29 were grown in spinner flasks on beads (Pierce) for three days, after which time a single cell suspension of RT4, J82 or control FHs-738B1 cells (Example 4) pre-labelled with the red fluorescent membrane linker PKH26 was added to the bead culture and allowed to form spheroids.

Using this model, the following characteristics of the tumour growth were measured, monitored, assessed, or estimated:

1. the invasiveness of the tumour bladder cells in the absence or the presence of given drugs using long term membrane linker tracers;
2. the growth rate and diameter of the tumour; the growth rate was assessed with the technique described above using the fluorescent membrane linker PKH26 and the Modfit software; the diameter of the tumour was monitored by imaging microscopy;
3. the molecular interactions between stromal and tumour tissues;
4. the expression of proliferation markers, for example PCNA, c-myc (used as prognosis markers in clinical usage) in the presence or absence of potential anti-proliferative drugs; after spheroid dissociation, the cells were immunolabeled and analysed by flow cytometry;
5. the expression of the connexins in both phenotypes as well as the level of cell-cell communication; this was done by immunolabeling and dye transfer, respectively, followed by flow cytometry analysis;

6. the cell adhesion; the expression of adhesion molecules was evaluated by immunolabeling and flow cytometry analysis; the adhesion itself was measured
using a commercially available kit.

In this model, any cancer cell phenotype can be substituted for bladder cell lines.

EXAMPLE 6

**Model of metastasis.**

Epithelial cells representative of the tissue where metastases are expected to develop are grown as monolayers on the lower surface of a modified insert in petri dishes. Small spheroids formed from tumour cells in spinner flasks are then seeded on the upper surface of the insert. The insert is then placed in its original position in the multiwell dishes. This model was developed using human normal lung fibroblasts CCD-37Lu (ATCC CRL 1496) as monolayers of normal cells and human embryonal carcinoma Tera1 (ATCC HTB 105) or Tera2 (ATCC HTB 106) as tumour cells. The cells forming the spheroids were pre-labelled with a membrane linker.

The following characteristics of the model were measured:

1. growth of both cell types
2. communication between cells
3. cell adhesion
4. cell invasion
5. cell differentiation

This model is particularly suitable for screening and testing of antimetastatic drugs.

EXAMPLE 7

**Model of senescence, apoptosis and tissue regeneration.**

This model was developed using embryonic stem cells (D3 strain), but normal cells of any origin can be used, either as self-forming spheroids or as spheroids growing on microcarrier beads. For monitoring the effect of senescence, a given percentage of a population of single cells in suspension was pre-treated with mitomycin at a concentration which blocks proliferation without killing the cells. The mitomycin-treated cells become progressively senescent and necrosis follows. The rest of the cell population was labelled with the fluorescent membrane linker PKH26. Both sub-populations were then mixed and allowed to form spheroids in appropriate medium in spinner flasks and the regeneration of the spheroids was followed by monitoring the proliferation of the neighbouring cells.

A similar model was designed for monitoring the effect of apoptosis. In this model, a given percentage of a population of single cells in suspension was loaded with a phototoxic compound (chloromethyl eosine diacetate: CMEDA). The rest of the cell population was labelled with the fluorescent membrane linker PKH26. Both sub-populations were then mixed and allowed to form spheroids in appropriate medium in spinner flasks. The spheroids are illuminated to induce cells death of CMEDA loaded cells and the regeneration of the spheroids was followed as mentioned above by monitoring the proliferation of the surviving cells.

This model can be customized for the study of the evolution of xenotransplants by using co-culture of stromal cells and progenitors of the desired phenotype grown as spheroids. Differentiation of the progenitors can be monitored by analysing specific antigens.

Original protocols were developed for this model to measure the effect of increasing the number of senescent, necrotic or apoptotic cells on the proliferative ability of neighbouring cells (pancreas, kidney, liver, heart).

This model is particularly useful for:

1. studying cell apoptosis in 3D
2. studying cell aging and necrosis in 3D
3. assaying drugs for tissue repair after trauma and ischemic damages.
4. testing drugs for tissue regeneration, especially for neuro-degenerative diseases
5. monitoring the evolution of xenotransplants with and without drug treatment
6. increasing the rate of recombination events in ES engineered cells for the production of transgenes.

In connection with the latter utility of the model, it was found that induction of apoptosis using the chloromethyl eosin diacetate (CMEDA) in a defined number of embryonic stem (ES) cells in spheroids (embryoid bodies) accelerates proliferation rate of live cells and shortens their cell cycle. The number of recombination events is relatively rare and was shown to depend upon the ES cell cycle duration. Therefore, the two conditions caused by the induction of cell death in the ES population lead to an increase in the number of recombinations. This model may also be used to increase the number of spontaneous mutations.

EXAMPLE 8

**Fitting theoretical functions to experimental data**

Fig. 7 through Fig. 10 show examples of application of the AD-JUST routine to experimental data analysis.

Fig. 7 shows the effect of a chemical (AGA) on the maintenance of a nonproliferative fraction of cells in 3D embryoid bodies (EB) formed of embryonic stem cells (curve ES-EBs + AGA) compared with the control (curve ES-EB) and with a hyperplastic cell line (curve F9-EBs). Theoretical functions

fitting the experimental data points are: reverse exponential (ES), negative exponential (F9) and reverse logistic (ES+AGA).

Fig. 8 shows the effect of AGA on the development of cell coupling in ES-EBs of two cell lines (ES and F9). Theoretical functions fitting the experimental data points are logistic functions with different parameters.

Fig. 9 and Fig. 10 show the kinetics of cell recruitment rate in the three EB types of Fig. 7 and the kinetics of cell coupling in 3D cell bodies of Fig. 8, respectively. The curves in Fig. 9 and 10 have been derived from the functions fitted to data points of Fig. 7 and 8, respectively.

The following equations represent the best fit for experimental data points in Figure 7:

$$F \quad e^{0 \quad t\ 0.} \quad 0 \quad r^2 \quad 0. \quad *$$

$$NPF_9 = -.066 - 02 - .031 \ (\text{gfc} = 959 \ *)$$

$$NPF_{ES} = 1.059 - e^{0.037t-2.804} \ (\text{gfc}\, r^2 = 0.994 **)$$

$$NPF_{ES+AGA} = 1 - \frac{1}{1.835 + e^{-0.563t+7.184}} + 0.445 \ (\text{gfc}\, r^2 = 0.959 **)$$

The following equations represent the best fit for experimental data points in Figure 8:

$$GJIC_{ES} = 99.21 - \frac{1}{0.0009 + e^{0.206t-4.652}} \ (\text{gfc}\, r^2 = 0.967 **)$$

$$GJIC_{F9} = 1.79 + \frac{1}{0.04 + e^{-3.311t+5.039}} \ (\text{gfc}\, r^2 = 0.999 **)$$

$$GJIC_{ES+AGA} = 101.71 - \frac{1}{0.007 + e^{0.216t-5.984}} \ (\text{gfc}\, r^2 = 0989 *)$$

where gfc $r^2$ : goodness of fit squared correlation; correlation significant at the 0.95 (*)
and the 0.99 (**) probability level.

EXAMPLE 9

**Simulating behavior of 3D cell aggregates using SIMCEL**

Gap junction intercellular communication (GJIC) is involved in several basic biological processes such as proliferation control, cell differentiation, organismic development, heart beat synchronization, astrocytes, neuron coupling and cancer. They also have potential implication in medical technologies (e.g. drug delivery through intracellular cell-to-cell diffusion and bystander effect in gene therapy).

The 3D *in vitro* models of the present invention may be used to investigate and test various chemical compounds (potential drugs, food additives, environmentally harmful chemicals, etc.) with respect to their effect on these biological processes. These investigations require fine kinetic analyses that are time consuming and expensive. Numerical experiments using simulation software can be a useful and sensible way to complement experimental techniques, thus saving time and money.

Using numerical experiments, certain features of a cell system can be predicted based on the level of cell coupling and using previous experimental data to set the values of parameters of the simulation model. Among these predictable features, the cycling cell recruitment from the resting pool, the proliferation index and the cell-to-cell intercellular diffusion are of special interest. The predictive capacity of SIMCEL-3D is illustrated in Table 3 and Fig. 11 and 12.

Table 3 shows a comparison of simulated and experimental data for 3 cell phases of the cell cycle and for 3 different cell lines. As the log-likelihood test

indicates, none of the real systems analyzed differ significantly from the simulated system.

Table 3. Comparison of simulated and experimental data for 3 cell phases of the cell cycle (cell %) in 3 different cell lines.

| Cells lines | G0/G1 | S | G2/M | Log-lik. |
|---|---|---|---|---|
| Human neuroblastoma (IMR 32) | 73.0 | 22.2 | 4.8 | NS |
| Stationary phase L6 rat myobalsts (L1) | 68.1 | 29.9 | 2.0 | NS |
| Stationary phase L6 rat myobalsts (L2) | 77.0 | 18.5 | 3.5 | NS |
| Simulated cell system | 73.9 | 21.9 | 4.2 | |

As an example, Figures 11 and 12 display the result of an numerical experiment performed with SIMCEL-3D. This simulation which ran over a time period of 290 time units (approximately 290 hours) was meant to predict the number of mitoses as a function of the G0-t cell fraction (transient resting cells with fully functional cell-cell communication channels. Both variables were shown to be dynamically related.

Although various particular embodiments of the present invention have been described hereinbefore for the purpose of illustration, it would be apparent to those skilled in the art that numerous variations may be made thereto without departing from the spirit and scope of the invention, as defined in the appended claims.